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Dihydrofolate Reductase from Kaposi's Sarcoma-Associated Herpesvirus

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the first human virus known to encode dihydrofolate reductase (DHFR), an enzyme required for nucleotide and methionine biosynthesis. We have studied the purified KSHV-DHFR enzyme *in vitro* and analyzed its expression in cultured B-cell lines derived from primary effusion lymphoma (PEL), an AIDS-associated malignancy. The amino acid sequence of KSHV-DHFR is most similar to human DHFR (hDHFR), but the viral enzyme contains an additional 23 amino acids at the carboxyl-terminus. The viral DHFR, overexpressed and purified from *E. coli,* was catalytically active *in vitro.* The K_m of KSHV-DHFR for dihydrofolate (FH_2) was 2.4 μM, which is significantly higher than the K_m of recombinant hDHFR (rhDHFR) for FH_2 (390 nM). K_m values for NADPH were similar for the two enzymes, about 1 μM. KSHV-DHFR was inhibited by folate antagonists such as methotrexate (K_i: 200 pM), aminopterin (K_i: 610 pM), pyrimethamine (K_i: 29 nM), trimethoprim (K_i: 2.3 μM), and piritrexim (K_i: 3.9 nM). In all cases, K_i values for these folate antagonists were higher for KSHV-DHFR than for rhDHFR. The viral enzyme was expressed at levels two- to tenfold higher than hDHFR in PEL cell lines as an early lytic cycle gene. KSHV-DHFR mRNA and protein appeared from 6 to 24 h after chemical induction of the KSHV lytic cycle. Epitope-tagged KSHV-DHFR and rhDHFR both localized to the nucleus of transfected cells, while other KSHV nucleotide metabolism genes localized to the cytoplasm. DHFR activity was not essential for viral replication in cultured PEL cells. Since hDHFR was not detectable in peripheral blood mononuclear cells (PBMCs), KSHV-DHFR may function to provide increased DHFR activity *in vivo* in infected cells that have little or none of their own enzyme.

INTRODUCTION

Kaposi's sarcoma (KS) is the neoplasm most frequently recognized among patients with AIDS (Goedert *et al.,* 1998). Using the technique of representational difference analysis, Chang *et al.* (1994) discovered herpesvirus-like sequences in KS lesions. These DNA sequences led to the identification of a novel herpesvirus, now known as Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8). KSHV is a gammaherpesvirus, related to Epstein–Barr virus (EBV) and *Herpesvirus saimiri* (HVS) (Weiss, 1996). The KSHV genome encodes several enzymes involved in nucleotide synthesis, including thymidine kinase (TK), thymidylate synthase (TS), ribonucleotide reductase (RR), and dihydrofolate reductase (DHFR) (Russo *et al.,* 1996). Virally encoded DHFRs have been identified only in T4 bacteriophage and the γ-two rhadinoviruses, including Rhesus rhadinovirus (RRV) (Searles *et al.,* 1999), HVS, and KSHV.

DHFR, an enzyme that is ubiquitous in nature, serves to maintain pools of reduced folate cofactors needed for one-carbon donations in *de novo* purine synthesis, thymidylate synthesis, and synthesis of the amino acid methionine. DHFR is responsible for reduction of dihydrofolate (FH_2), formed in the TS reaction, to tetrahydrofolate (FH_4), allowing continued function of the cyclic pathway which produces thymidylate for DNA synthesis. In prokaryotes, which cannot take up exogenous folates, DHFR is also an essential element of the pathway for *de novo* folate biosynthesis. Intensive study of DHFR has led to the development of inhibitors which are widely used pharmacological agents in the treatment of cancer, as immunosuppressive agents and as antibiotics against bacterial and protozoal infections.

The genomes of all human herpesviruses encode some enzymes involved in nucleotide metabolism. RR is encoded by all human herpesviruses. TK is encoded by the alpha and gammaherpesviruses. TS and DHFR are encoded by some members of the human herpesvirus group; for example, varicella zoster virus (VZV) and KSHV are the only members known to encode TS. The functions of these viral enzymes in the viral life cycles and pathogenesis are not yet clear. None of the viral enzymes has been shown to be required for herpesvirus replication in tissue culture (Pyles *et al.,* 1992). For example, the enzymes TK, RR, and deoxyuridine triphosphate hydroxylase (dUTPase) are not essential for repli-

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TABLE 1

Comparison of Amino Acid Identity and Similarity of KSHV-DHFR to DHFRs of Six Other Organisms

DHFR	KSHV[a]	T4 bacteriophage	*P. falciparum*	*E. coli*	*S. cerevisiae*	HVS[b]
Human	50/66	24/37	34/46	35/55	35/45	83/87
HVS	48/58	24/36	31/43	33/51	33/45	
S. cerevisiae	30/43	29/43	38/49	28/40		
E. coli	28/48	30/39	25/40			
P. falciparum	28/35	25/34				
T4 bacteriophage	14/28					

Note. The *P. falciparum*-DHFR sequence represented is the DHFR domain of the bifunctional DHFR-thymidylate synthase enzyme.

[a] Numbers are % identity/% similarity.

[b] HVS = *Herpesvirus saimiri*.

cation of herpes simplex virus 1 (HSV-1) in culture (Kit and Dubbs, 1963; Fisher and Preston, 1986; Goldstein and Weller, 1988; Daikoku *et al.,* 1991); similarly, elimination of DHFR from the genome of HVS does not appear to affect viral replication or the immortalization of T-cells in culture (Desrosiers *et al.,* 1985, 1986). Nevertheless, these enzymes presumably play some crucial role in viral pathogenesis *in vivo*. For example, HSV-1 TK seems to be important for reactivation from latency (Field and Wildy, 1978; Coen *et al.,* 1989; Tenser *et al.,* 1989; Jacobson *et al.,* 1993).

The viral nucleotide metabolism enzymes have proved to be susceptible targets for antiviral therapy (Elion, 1993). HSV1-TK has been shown to catalyze phosphorylation of nucleoside analogs such as acyclovir (ACV) and ganciclovir (GCV). This results in selective phosphorylation of ACV in virus-infected cells, with consequent misincorporation of the fraudulent, replication-terminating nucleotide into the viral DNA. Recently, three groups have reported that KSHV is sensitive to antiviral drugs such as cidofovir and GCV that work by this mechanism (Kedes and Ganem, 1997; Medveczky *et al.,* 1997; Cannon *et al.,* 1999). HSV1-RR inhibitors have also been used in conjunction with ACV to decrease the competing dGTP pools and thus increase the probability of ACV phosphorylation and misincorporation (Spector *et al.,* 1992). The viral enzymes have also found applications in gene therapy. HSV1-TK has also been used as a transfected "suicide" agent to facilitate tumor-cell killing by ganciclovir (Klatzmann *et al.,* 1998). Thus drugs that target the viral nucleotide metabolism genes may be useful in treatment or prevention of KS.

In this report, we describe an initial study of KSHV-DHFR, the first example of this enzyme encoded by a human virus. An ultimate goal is to understand the role KSHV-DHFR plays in viral replication and pathogenesis and its possible value as a target for antiviral or antitumor chemotherapy. We have compared the amino acid sequence of KSHV-DHFR to those of other species. We have expressed KSHV-DHFR in bacteria and purified the protein. This allowed study of KSHV-DHFR kinetics and its inhibition by various folate antagonists, including methotrexate (MTX), trimethoprim (TMP), pyrimethamine (PYR), aminopterin (AMT), and piritrexim (PTX). We have extensively studied KSHV-DHFR and human DHFR (hDHFR) mRNA and protein expression in tissue culture. Using epitope-tagging methods, we have found that KSHV-DHFR and recombinant hDHFR (rhDHFR) localized primarily to the nucleus, a finding that might provide some insight into the debate over the intracellular localization of the process of nucleotide metabolism (Prem veer Reddy and Pardee, 1980; Leeds *et al.,* 1985). We have noted that levels of cellular DHFR are intrinsically low in peripheral blood mononuclear cells (PBMCs), which are thought to be susceptible to KSHV infection *in vivo* (Ambroziak *et al.,* 1995; Blasig *et al.,* 1997). Viral DHFR may therefore function to assist KSHV growth in resting cells that have not been activated into the cell cycle.

RESULTS

Amino acid sequence analysis

KSHV-DHFR is a 210-amino-acid (a.a.) protein with a predicted mass of 24 kDa. Amino acid sequence comparisons showed that the KSHV-DHFR sequence is most similar to the a.a. sequence of hDHFR, with which it shares 50% identity and 66% similarity (Table 1). This similarity is considerably less than the identity between HVS-DHFR and hDHFR (83%). At the nucleotide level, the percentage identity between KSHV-DHFR and hDHFR is 52%. Again, this is less than the nucleotide identity between HVS-DHFR and hDHFR (76%). Only the first 187 of the 210 amino acids of KSHV-DHFR can be aligned with hDHFR or other DHFRs (Fig. 1). The 23-a.a. "tail" is unrelated to any known a.a. sequence and its function, if any, is unknown. Bacteriophage T4 is the only virus that is not a gammaherpesvirus known to encode a DHFR. Only 165 out of 193 amino acids of bacteriophage T4 DHFR can be aligned with other DHFRs (Bernstein and Bernstein, 1989). The sequence identity/similarity between KSHV-DHFR and T4 DHFR is only 14%/28%. Table

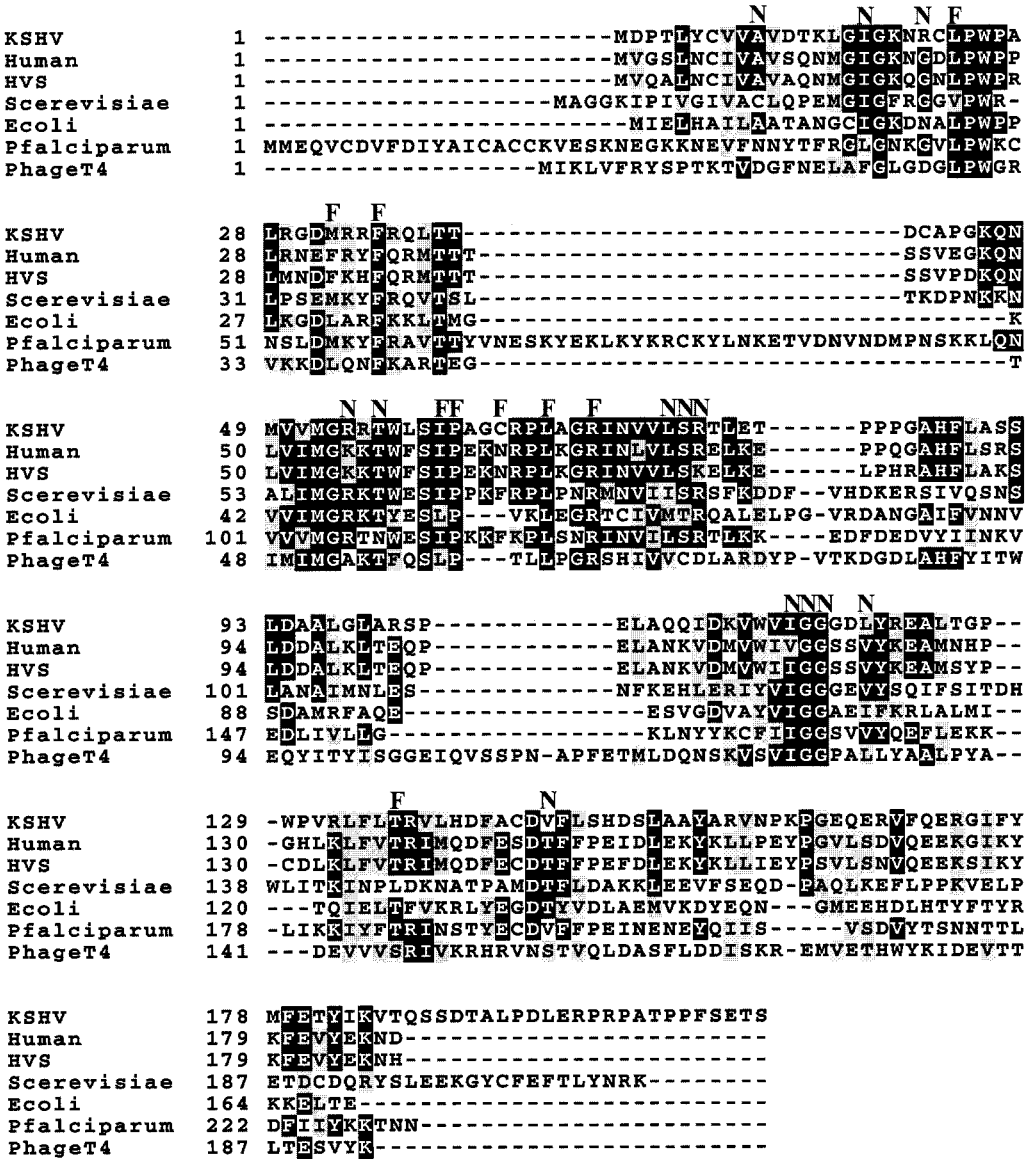

FIG. 1. Amino acid sequence analysis of DHFR. This figure compares the KSHV-DHFR amino acid sequence to those of several other DHFRs. Black boxes show an identity between sequences; shaded boxes show a similarity. F = amino acid involved in dihydrofolic acid binding or reduction. N = amino acid involved in NADPH binding or oxidation. HVS = *Herpesvirus saimiri*. The *P. falciparum*-DHFR sequence represented is the DHFR domain of the bifunctional DHFR-thymidylate synthase enzyme.

1 shows the identity/similarity between KSHV-DHFR and the DHFRs from the organisms whose DHFR a.a. sequences are shown in Fig. 1. KSHV-DHFR is most similar to DHFR from eukaryotic cells and viruses but also shares significant similarity with prokaryotic cellular and viral DHFRs, particularly in the active site (Table 1 and Fig. 1).

A more specific comparison of amino acids known to be involved functionally in binding of dihydrofolic acid (FH_2) and NADPH by both prokaryotic and eukaryotic DHFRs revealed that KSHV-DHFR had identical or similar amino acids at nearly all of these binding sites (Fig. 1). However, KSHV-DHFR has an aspartic acid (D) at a.a. 31, where the corresponding a.a. in hDHFR is a glutamic acid (E). This difference may be interesting because this is the amino acid believed to be responsible for proton transfer to FH_2 (Brown and Kraut, 1992). Studies of the E31D mutant of rhDHFR showed an increase in K_m for FH_2 and NADPH (Blakley, 1995).

Expression and activity of KSHV-DHFR

The gene for KSHV-DHFR was cloned and expressed in *E. coli* and the protein purified by affinity chromatography (Fig. 2A). KSHV-DHFR purified from the extracts was found to be catalytically active *in vitro* in a classical assay that measures the conversion of FH_2 to FH_4 and NADPH to $NADP^+$ (Fig. 2B). Analysis of the steady-state

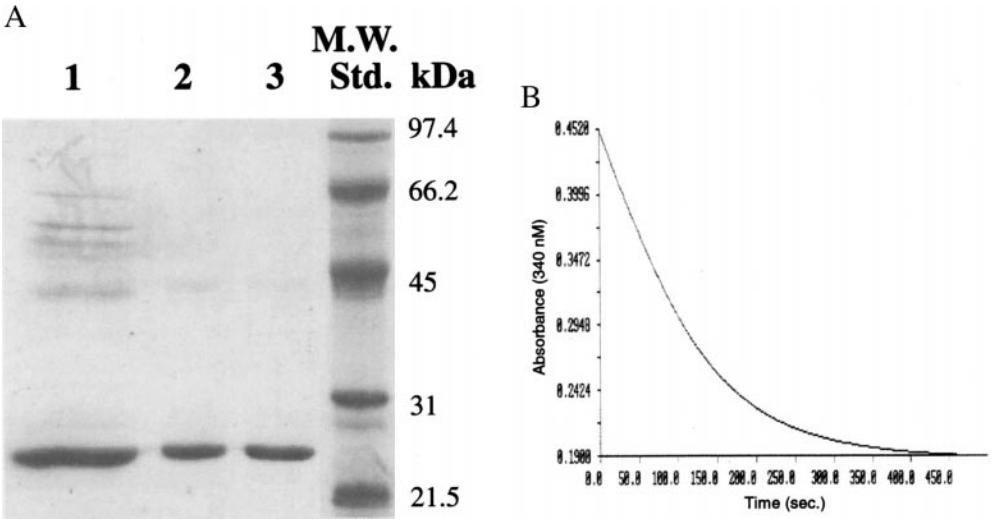

FIG. 2. Purification and activity of KSHV-DHFR. (A) Coomassie blue-stained SDS–PAGE containing the ultimate and penultimate steps in purification of KSHV-DHFR, which was expressed as a fusion protein with an intein self-cleaving element at the C-terminal end of KSHV-DHFR and a chitin-binding domain at the C-terminus of the intein element. Lane 1 shows 60 μg of protein obtained after a chitin bead affinity column purification and autocleavage. Lanes 2 and 3 show the two purest fractions after one run over a G-75 Sephadex gel filtration column (25 μg per lane). This method of purification typically yielded 4 mg of pure protein per liter of *E. coli*. (B) A representative KSHV-DHFR activity assay reaction depicting the decrease in absorbance at 340 nM resulting from the reduction of dihydrofolic acid to tetrahydrofolic acid and the oxidation of NADPH to $NADP^+$. Three micrograms of protein from the final pool of purified protein (Lane 3 on the gel illustrated in A) was used in this particular reaction.

kinetics of KSHV-DHFR revealed that the K_m for FH_2 was 2.4 μM, significantly higher than the value of 390 nM we determined for rhDHFR (Table 2). A more accurate determination of the K_m was hindered by substrate inhibition that was observed at 8 μM FH_2 for the viral enzyme and 1 μM for rhDHFR (data not shown). K_m values for NADPH were about 1 μM for both the viral and human enzyme (Table 2). The K_m determination for NADPH was monophasic, in contrast to the biphasic K_m of NADPH reported previously for rhDHFR (Appleman *et al.,* 1990). Interestingly, the rhDHFR E30D mutant was also reported to have a monophasic K_m for NADPH (Blakley, 1995). The turnover number, or k_{cat}, for KSHV-DHFR was about half the value determined for rhDHFR (Table 2). Division of k_{cat} by K_m gives a measure of the overall catalytic efficiency of the enzymes, with higher numbers being indicative of a more efficient enzyme. From the k_{cat}/K_m numbers in Table 2 it can be concluded that rhDHFR is at least 14-fold more efficient than KSHV-DHFR under our assay conditions.

TABLE 2

Steady-State Kinetic Values of KSHV-DHFR, ntKSHV-DHFR, and rhDHFR

	KSHV-DHFR	ntKSHV-DHFR[a]	rhDHFR[b]	rhDHFR (published)[c]
K_m FH_2 (μM)	2.4 ± 0.5	2.2 ± 0.5	0.4 ± 0.1	0.12
K_m NADPH (μM)	0.95 ± 0.12	1.3 ± 0.2	1.1 ± 0.1	0.16–4.2
k_{cat} (s^{-1})	7.0 ± 0.7	4.8 ± 0.1	16.8 ± 0.4	11 ± 2
k_{cat}/K_m (s^{-1} μM^{-1})	2.9 ± 0.6	2.2 ± 0.5	42 ± 11	91.7

[a] ntKSHV-DHFR = KSHV-DHFR, 23-a.a. C-terminal truncation mutant.
[b] rhDHFR = recombinant human DHFR.
[c] From Appleman *et al.,* 1990.

To further evaluate the kinetic similarity between KSHV-DHFR and eukaryotic DHFRs, experiments were performed to determine whether KSHV-DHFR exhibited a property attributed only to prokaryotic DHFRs known as slow enzyme hysteresis (for a review, see Appleman *et al.,* 1989). Slow enzyme hysteresis arises as a result of a slow transition between the nonbinding and binding conformations of an enzyme. DHFRs that exhibit slow enzyme hysteresis (i.e., prokaryotic DHFRs) are termed classical DHFRs. Vertebrate DHFRs also exhibit a change in conformation related to substrate binding, but on a time-scale 1000-fold faster than invertebrate DHFRs. These enzymes are termed nonclassical (Appleman *et al.,* 1989). Slow enzyme hysteresis was tested for by looking for a difference in reaction velocity between reactions that contained enzyme that was or was not preincubated with substrate (Appleman *et al.,* 1989). We found that KSHV-DHFR does not exhibit slow enzyme hysteresis and thus, like vertebrate DHFRs, may be termed a nonclassical DHFR (data not shown).

Another property unique to vertebrate DHFRs is the existence of dual pH optima; prokaryotic DHFRs have one optimum (Blakley, 1969). The pH profile for KSHV-DHFR was determined by testing identical reactions across a broad range of pHs (4.5–9). The viral DHFR exhibited dual pH optima at pH 4.5 and 8 (data not shown). This is further evidence that the viral DHFR is kinetically similar to vertebrate DHFRs.

TABLE 3

Comparison of the Sensitivity of KSHV-DHFR and Recombinant Human DHFR to Five Known Inhibitors of DHFR

Drug	KSHV-DHFR[a]	rhDHFR[b]	rhDHFR (published)
Methotrexate	200 ± 18 pM	9 ± 2 pM	3.4 ± 1.2 pM[c]
Aminopterin	610 ± 20 pM	ND[d]	14.6 ± 0.1[c], 1.8 pM[e]
Piritrexim	3.9 ± 0.4 nM	8 ± 2 pM	7.7 ± 1.2 pM[f]
Pyrimethamine	27 ± 3 nM	1.0 ± 0.2 nM	1.15 ± 0.06 nM[f]
Trimethoprim	2.3 ± 0.2 μM	0.81 ± 0.02 μM	0.96 ± 0.3 μM[c]

[a] Numbers are the K_i values.
[b] rhDHFR = recombinant human DHFR.
[c] From Appleman *et al.,* 1988b.
[d] ND = not determined.
[e] From Margosiak *et al.,* 1993.
[f] From Ercikan-Abali *et al.,* 1996.

A truncation mutant of KSHV-DHFR, lacking the C-terminal 23-a.a. region, was also constructed (ntKSHV-DHFR). The steady state properties of ntKSHV-DHFR are shown in Table 2. The K_m and k_{cat} values for the truncation mutant were not significantly different from those of the wild-type enzyme.

Inhibition analysis of KSHV-DHFR

K_i values for five known inhibitors of DHFR were obtained using both KSHV-DHFR and rhDHFR (Table 3). The K_i values determined for KSHV-DHFR were higher than those for rhDHFR in all cases. The K_i determination of AMT for KSHV-DHFR is shown in Fig. 3. The curvature of the plotted line indicates that AMT is not a "stoichiometric" inhibitor of KSHV-DHFR as it is known to be for hDHFR (Ercikan *et al.,* 1993). In the case of a stoichiometric inhibitor, such as MTX, PTX, or AMT for hDHFR, the velocity versus [inhibitor] plot is a straight line until the concentration of inhibitor equals the concentration of enzyme, which results from the enzyme's being titrated out of action through extremely tight binding of the inhibitor (Werkheiser, 1960). Thus stoichiometric inhibitors can be used to determine the amount of enzyme in solution (Bertino *et al.,* 1970). None of the inhibitors tested proved to be a stoichiometric inhibitor of KSHV-DHFR. Taken together, these preliminary studies did not identify an inhibitor with selective activity against KSHV-DHFR.

Comparison of expression of KSHV-DHFR and hDHFR mRNA in primary effusion lymphoma (PEL) cell lines

The kinetic class of KSHV lytic cycle genes has been determined in cultured PEL cell lines that have been treated with chemicals that induce the viral lytic cycle (Sun *et al.,* 1999). Nicholas *et al.* (1997) reported the appearance of a 1.2-kb transcript of the viral DHFR gene in the HBL6 cell line, which contains both the KSHV and EBV genomes, 12 h after induction of the lytic cycle. We examined the expression kinetics of the viral DHFR gene in the BC-1 PEL cell line, which also contains both EBV and KSHV, and in the PEL cell line HH-B2, established in our laboratory, which is infected only with KSHV (Fig. 4A). We detected a 1.1-kb transcript using a single-stranded, oligonucleotide probe specific for KSHV-DHFR mRNA. The 1.1-kb KSHV-DHFR mRNA appeared 13 h after treatment with butyrate and was present until 48 h after chemical induction (Fig. 4A and data not shown). When the same blots were probed with an oligonucleotide complementary to hDHFR, the 3.8-kb hDHFR transcript was present until 24 h following chemical induction, after which time it became undetectable (Chen *et al.,* 1984). The same experiments were also carried out using the cell line HH514–16 (Cl-16), a Burkitt-lymphoma derivative, which is infected only with EBV, and the B-lymphoma cell line BJAB, which is not virally infected. KSHV-DHFR mRNA was not detected in either of these cell lines (data not shown). In both these cell lines, a decrease in the amount of hDHFR transcript, quantitatively similar to that seen in the KSHV positive PEL cell lines, was seen 24 h after treatment with butyrate (data not shown).

The relative increase in KSHV-DHFR mRNA and the relative decrease in hDHFR mRNA up to 48 h after chemical induction of the lytic cycle, were quantified using a phosphorimager (Figs. 4B and 4C). Treatment with butyrate caused a more than 30-fold increase in the signal of KSHV-DHFR mRNA in the BC-1 cell line and a sixfold increase in the KSHV-DHFR signal in the HH-B2 cell line (Fig. 4B). Conversely, treatment with butyrate resulted in a 27-fold decrease in the signal of hDHFR mRNA in BC-1 cells and a 63-fold decrease in HH-B2 cells as compared to a sample that was not treated with drugs.

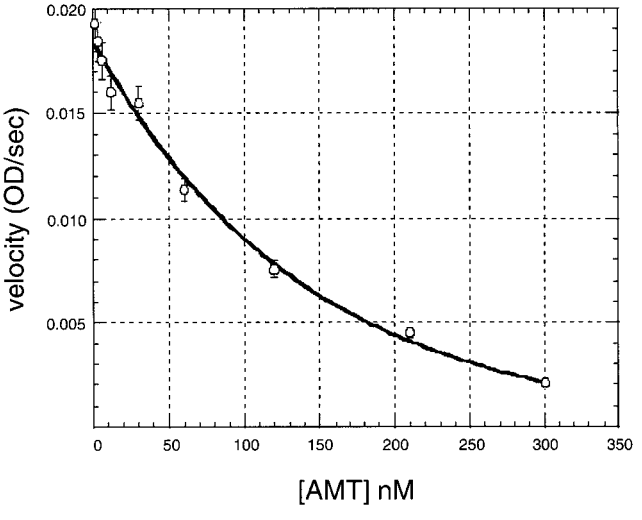

FIG. 3. Determination of the K_i of aminopterin for KSHV-DHFR. Varying concentrations of AMT (~1–300 nM) were added to the standard DHFR activity assay to generate an inhibition curve. K_i was determined by extrapolation to this curve.

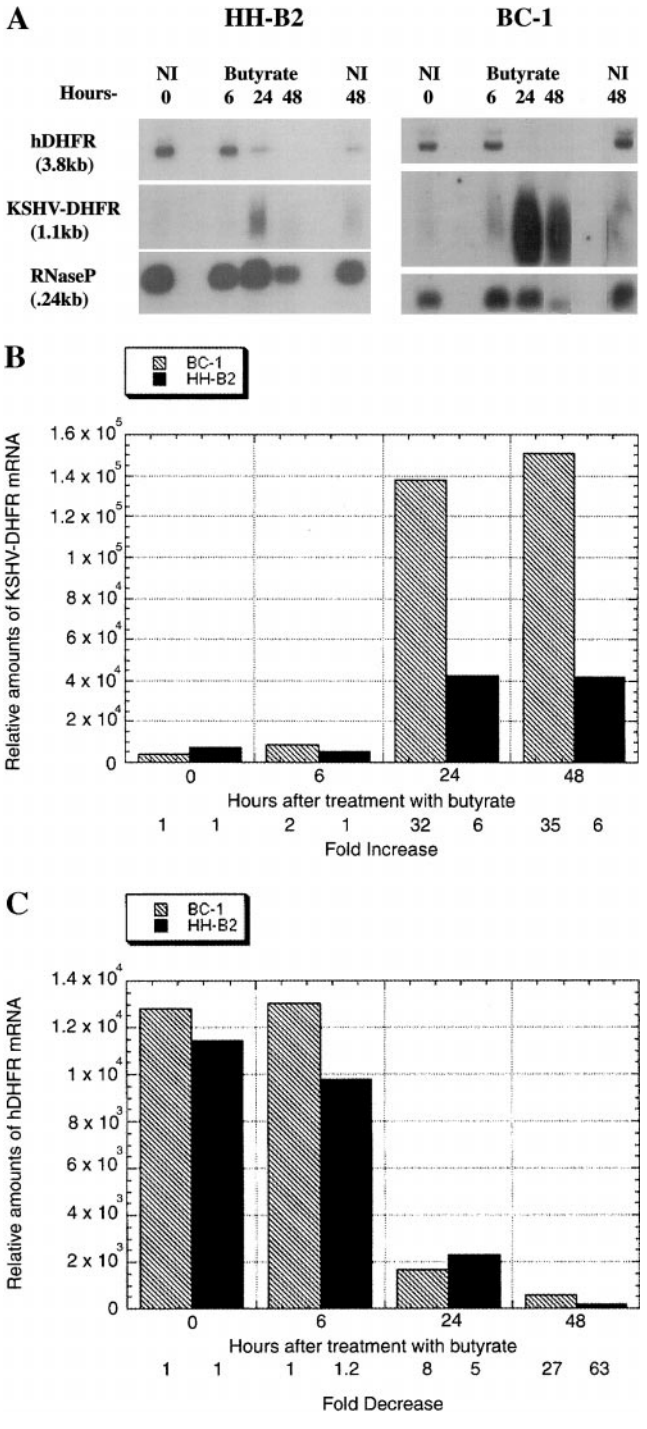

FIG. 4. Comparison of KSHV-DHFR and hDHFR mRNA after treatment of two primary effusion lymphoma (PEL) cell lines with butyrate. (A) Northern blot illustrating the expression of human and viral DHFR transcripts over a 48-h time period after treatment of the BC-1 and HH-B2 PEL cell lines with butyrate. The H1 component of RNase P was used to control for loading. NI = noninduced. (B) Quantitation of the relative amounts of KSHV-DHFR mRNA following treatment with butyrate. (C) Quantitation of the relative amounts of human DHFR mRNA following treatment with butyrate. The relative amounts of mRNA were determined by phosphorimaging of the data in panel A. Some lanes in panel A were rearranged to preserve continuity in the presentation of data from experiments with the two cell lines.

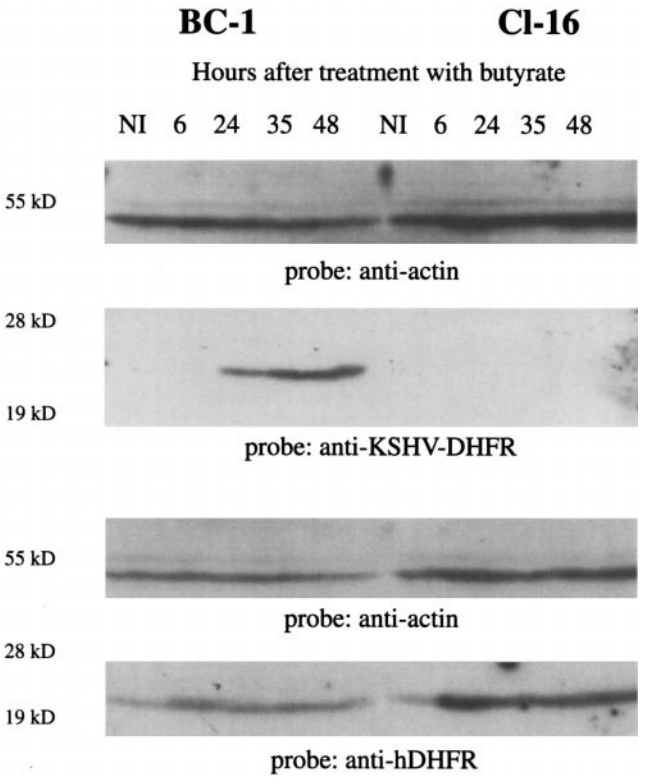

FIG. 5. Comparison of KSHV-DHFR and hDHFR protein in two B-cell lymphoma cell lines. Shown are Western blots probed with monospecific polyclonal rabbit antibodies to KSHV-DHFR, hDHFR, and β-actin. The blots illustrate the levels of KSHV-DHFR and hDHFR over a 48-h time course following treatment of the BC-1 PEL cell line and Cl-16 Burkitt lymphoma cell line with butyrate. NI = noninduced.

Other chemical agents known to activate the KSHV lytic cycle were also shown to induce expression of KSHV-DHFR mRNA. For example, tetradecanoyl phorbol acetic acid (TPA) induced viral DHFR mRNA expression in the BCBL-1 cell line and trichostatin A (TSA), a histone deacetylase inhibitor, activated KSHV-DHFR mRNA expression in the BC-1 cell line (data not shown).

Expression of KSHV-DHFR and hDHFR in PEL cell lines

To demonstrate that KSHV-DHFR protein was expressed in infected cells, we developed polyclonal antibodies to KSHV-DHFR by immunizing rabbits with the viral enzyme that was produced in *E. coli* and purified (Fig. 2A). The antibody to viral DHFR did not cross-react with rhDHFR that was expressed and purified from *E. coli* (data not shown). Moreover, the rabbit antiviral DHFR antibody did not detect hDHFR that was present in cultured B-cell lines (Fig. 5).

We determined the relative kinetics of expression of viral and cellular DHFR protein in a series of cultured B-lymphoma cell lines, including BC-1, HH-B2, BCBL-1, BC-3, Cl-16, and BJAB (Fig. 5, Table 4, and data not shown). Immunoblots prepared from untreated cell ex-

TABLE 4

Estimation of the Number of KSHV-DHFR and hDHFR Molecules Per Cell

KSHV-DHFR

	Copies per cell[a]			
			With correction, assuming 25% induction[b]	
Cell line (virus)	48-h NI	48-h but.	48-h NI	48-h but.
BC-1 (KSHV/EBV)	5×10^3	1.6×10^5	5×10^3	6.4×10^5
HH-B2 (KSHV)	6×10^3	1×10^4	6×10^3	4×10^4
BCBL-1 (KSHV)	4×10^3	2×10^4	4×10^3	8×10^4
BC-3[c] (KSHV)	7×10^3	1.6×10^5	7×10^3	6.4×10^5

Human DHFR

	Copies per cell[a]	
Cell line (virus)	48-h NI	48-h but.
BC-1 (KSHV/EBV)	5×10^4	6×10^4
HH-B2 (KSHV)	2×10^4	2×10^4
BCBL-1 (KSHV)	3×10^4	4×10^4
BC-3[c] (KSHV)	3×10^4	4×10^4
CL-16 (EBV)	1×10^4	4×10^4
BJAB (none)	1×10^4	4×10^4

Note. The data for all cell lines except the BC-3 cell line was obtained 48 h after subculture in the presence or absence of butyrate.

[a] NI = noninduced; but. = butyrate-treated.

[b] The corrected values assume that approximately 25% of the cells were induced into the lytic cycle.

[c] The BC-3 cell line was induced with TPA and butyrate.

tracts or from extracts of chemically induced cells were reacted with antibodies to KSHV-DHFR and hDHFR. KSHV-DHFR was not detected in cells, such as CI-16 and BJAB, which lack the KSHV genome (Fig. 5 and data not shown). In PEL cell lines containing KSHV, viral DHFR was invariably induced 24 h after chemical treatment. Immunoreactive, viral protein persisted for at least 48 h. The amount of viral DHFR detected in chemically induced cells varied among the PEL cell lines (Table 4). Equal amounts of hDHFR were detected in KSHV-negative and KSHV-positive cell lines. The abundance of hDHFR was only minimally affected by chemical treatment (Fig. 5, Table 4).

In cultures treated with butyrate, hDHFR protein was present in the cell extracts even after the hDHFR mRNA was no longer detectable (compare Figs. 4A and 5). hDHFR protein is known to have a long half-life of between 11 and 20 h (Jackson and Huennekens, 1973; Domin *et al.,* 1982). Therefore hDHFR produced in the first 20 h after butyrate treatment is likely to be present at 48 h.

Consistent with the mRNA results, KSHV-DHFR protein expression was also increased following treatment with a number of other inducing agents including TPA and TSA, which are known to activate the KSHV lytic cycle (data not shown).

Quantitation of KSHV-DHFR and hDHFR

To estimate the amount of KSHV-DHFR per cell in uninduced and lytically induced extracts, we performed phosphorimage analysis of immunoblots, comparing the signal from cell extracts with the signal from known amounts of purified viral and human DHFR protein (Table 4). The amount of KSHV-DHFR in uninduced cultures, from 4 to 7 $\times$ 10^3 molecules per cell, was remarkably similar among all the KSHV-infected PEL cell lines tested. Forty-eight hours after chemical induction of the lytic cycle, there was an increase in viral DHFR content in all the PEL cell lines. The BC-1 and BC-3 cell lines contained the highest amounts of viral enzyme, almost 10-fold the amounts detected in the BCBL-1 or HH-B2 cell lines. If it is assumed that about 25% of cells were chemically induced into the lytic cycle, viral DHFR content per cell rose from a minimum of sixfold (in HH-B2 cells) to a maximum of 128-fold (in BC-1 cells).

The copy number of hDHFR varied from 1 to 5 $\times$ 10^4 molecules per cell in the six cell lines tested (Table 4). This number increased about fourfold following butyrate treatment of the two KSHV-negative cell lines, but did not measurably change in the KSHV-positive cells. Comparing the level of viral and cellular DHFR in chemically treated cultures, there was at least twofold as much viral DHFR as cellular DHFR in the minimally responsive HH-B2 and BCBL-1 cell lines and more than 10-fold as much viral as cellular DHFR per cell in the maximally responsive BC-1 and BC-3 cell lines.

To compare the amount of hDHFR estimated to be present in the cultured B-lymphoma cells with the

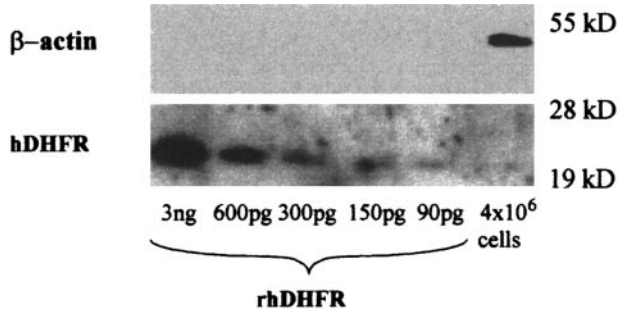

FIG. 6. Amount of hDHFR in human peripheral blood mononuclear cells (PBMC) depleted of CD-3 positive T-lymphocytes. This immunoblot, probed with antiserum to hDHFR, compares the level of hDHFR in a sample of 4 $\times$ 10^6 CD3-depleted human PBMCs with known amounts of recombinant hDHFR. The secondary probe was ^{125}I-labeled protein A. The blot was exposed for 14 days. Ninety picograms of hDHFR is roughly equivalent to 650 copies/cell in a 4 $\times$ 10^6 cell sample. The blot was probed with mouse monoclonal antibody to human β-actin to control for loading of the cell sample.

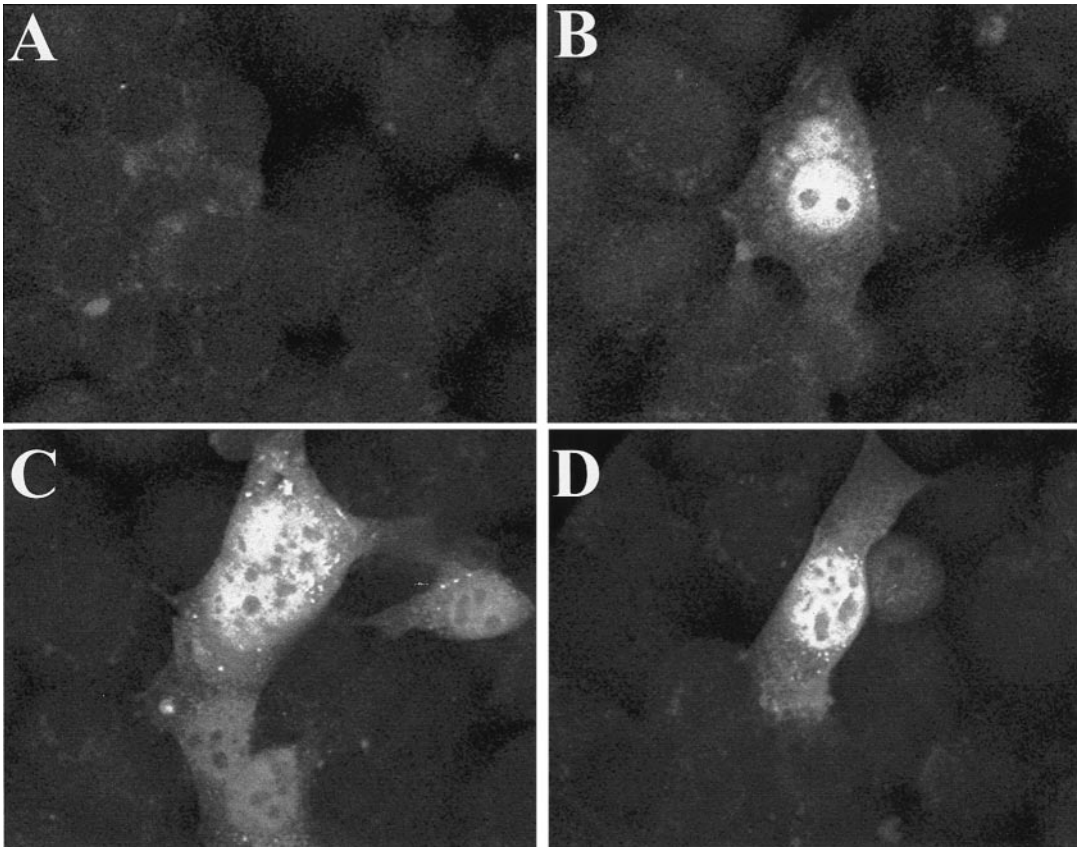

FIG. 7. Intracellular localization of KSHV-DHFR, rhDHFR, and KSHV-TS. CHO cells were transfected with vector alone or with vector containing KSHV-DHFR, rhDHFR, or KSHV-TS with an N-terminal flag tag. Cells were fixed and incubated with a mouse monoclonal antibody to the M2 flag-tag epitope, which was detected with a rabbit antimouse IgG TRITC conjugated secondary antibody. (A) Cells shown were transfected with vector alone. (B–D) Cells transfected with KSHV-DHFR/flag tag. (E) Cells transfected with rhDHFR/flag tag. (F, G) Cells transfected with KSHV-TS/flag tag.

amount of hDHFR present in normal B-cells, we analyzed a sample of PBMCs that were depleted of CD3-positive T-cells, leaving mainly B-cells and monocytes, the cell types in which KSHV has been detected in the blood (Ambroziak *et al.,* 1995; Blasig *et al.,* 1997). This experiment (Fig. 6) showed that hDHFR was undetectable in this cell population. The smallest amount of hDHFR that could be detected was 90 pg, which corresponds to approximately 650 copies per cell. Thus, B-cell primary effusion lymphomas grown in tissue culture contain at least 15- to 77-fold more hDHFR than peripheral blood B-cells and monocytes.

Intracellular localization of KSHV-DHFR, rhDHFR, and KSHV-TS, -TK, -RRα, and -RRβ

The next series of experiments explored two questions: (1) whether viral and cellular DHFR were found in the same or different cellular compartments and (2) whether other enzymes involved in nucleotide metabolism encoded by KSHV were present in the same cellular compartment as DHFR. When expressed with an N-terminal flag tag, both KSHV-DHFR and rhDHFR were localized almost exclusively to the nuclei of the Chinese hamster ovary (CHO) cells and excluded nucleoli (Figs. 7B–7D and 7E, respectively). Although there were a few cells also containing varying amounts of speckled fluorescence in the cytoplasm, there were no fluorescent cells that lacked nuclear staining for either human or viral DHFR. These results were confirmed in COS cells and in the HH-B2 PEL cell line (data not shown). KSHV-DHFR and rhDHFR protein expressed as a C-terminal green-fluorescent fusion protein were also localized to the nucleoplasm (data not shown). The 23-a.a. C-terminal KSHV-DHFR truncation mutant was also localized exclusively to the nucleus.

By contrast, KSHV-TS appeared to be localized completely to the cytoplasm (Figs. 7F and 7G), as did KSHV-TK and KSHV-RRα (data not shown). KSHV-RRβ was the only gene tested that was seen to localize to the cytoplasm in some cells and to the nucleus of other cells (data not shown). The localizations of KSHV-TS, -TK, -RRα, and -RRβ were similar in CHO cells, COS cells, and in the HH-B2 PEL cell line. The localization of these four enzymes was similar, whether constructs contained an N-terminal myc-epitope tag or an N-terminal flag tag (data not shown).

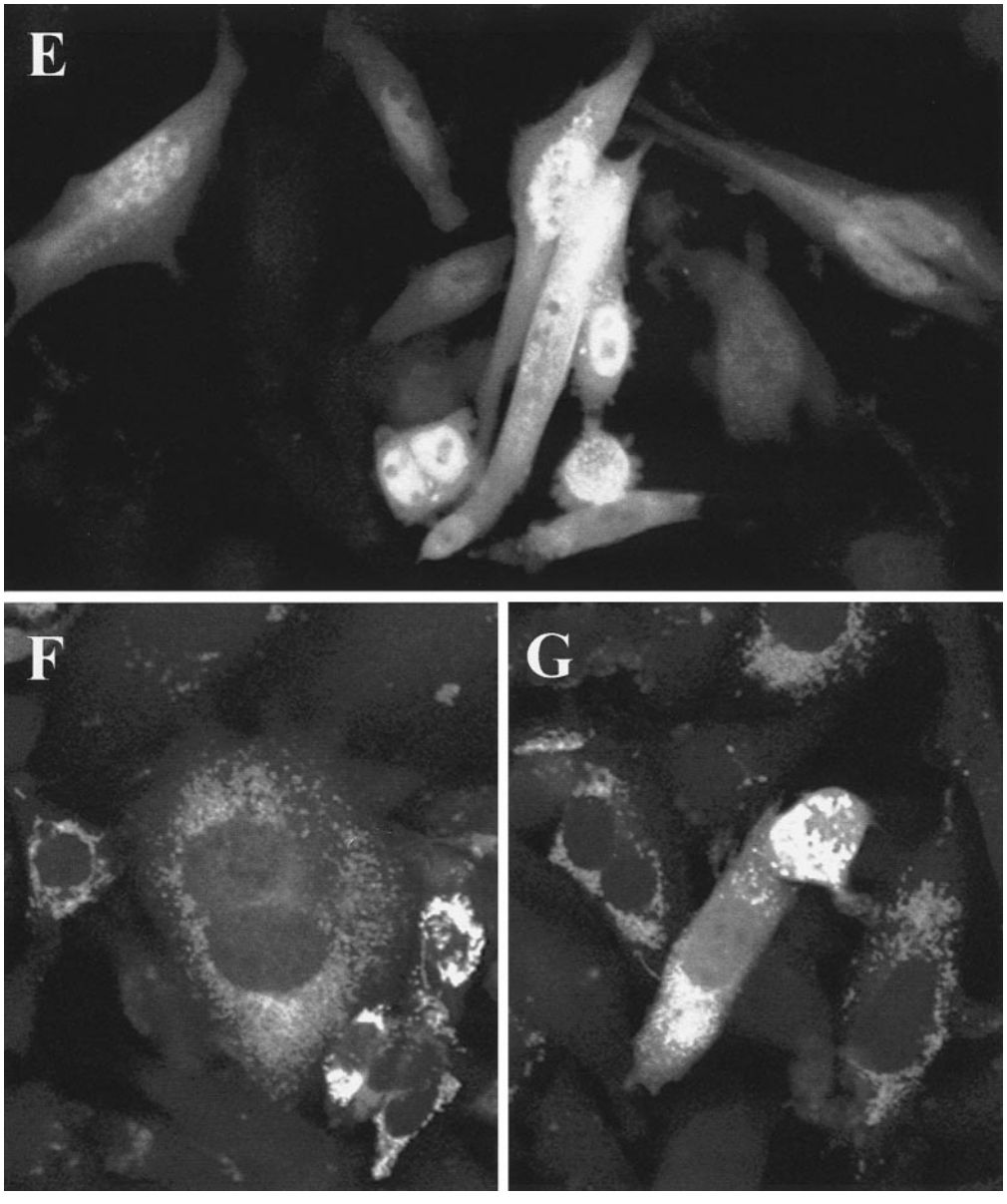

FIG. 7—*Continued*

Cotransfection of flag-tagged KSHV-DHFR, a nuclear product, and myc-tagged KSHV-TS, a cytoplasmic product, into CHO cells allowed for double-staining, which showed no evidence of association of the two enzymes (data not shown).

Effects of AMT on KSHV replication in culture

To determine whether DHFR activity is necessary for KSHV replication, BC-3 cells were first adapted to growth in media containing hypoxanthine (H), aminopterin (A), and thymidine (T) (HAT). AMT inhibited DHFR activity in HAT-adapted BC-3 cells. When such cells were deprived of H and T, and exposed to AMT, they failed to proliferate (Fig. 8A). HAT-adapted BC-3 cells, subcultured in medium with no supplement, with HT only, with HAT, or with AMT, were treated with TPA and butyrate to induce the KSHV lytic cycle. As an index of viral replication, the amount of viral DNA was measured by Southern blotting followed by phosphorimaging (Fig. 8B). As an indirect measure of viral replication the amount of sVCA, a late protein, was detected by immunoblotting (Fig. 8C). Treatment of HAT-adapted BC-3 cells with TPA and butyrate caused an increase in viral DNA content. A similar increase in viral DNA content was observed whether or not the cells were provided with H and T and whether or not they were treated with 400 nM AMT (Fig. 8B). Similarly, under all conditions there was an increase in the content of sVCA, a late gene whose expression is dependent on viral DNA replication (Lin *et al.,* 1997; Chan *et al.,* 1998). Although there were slight decreases in viral DNA con-

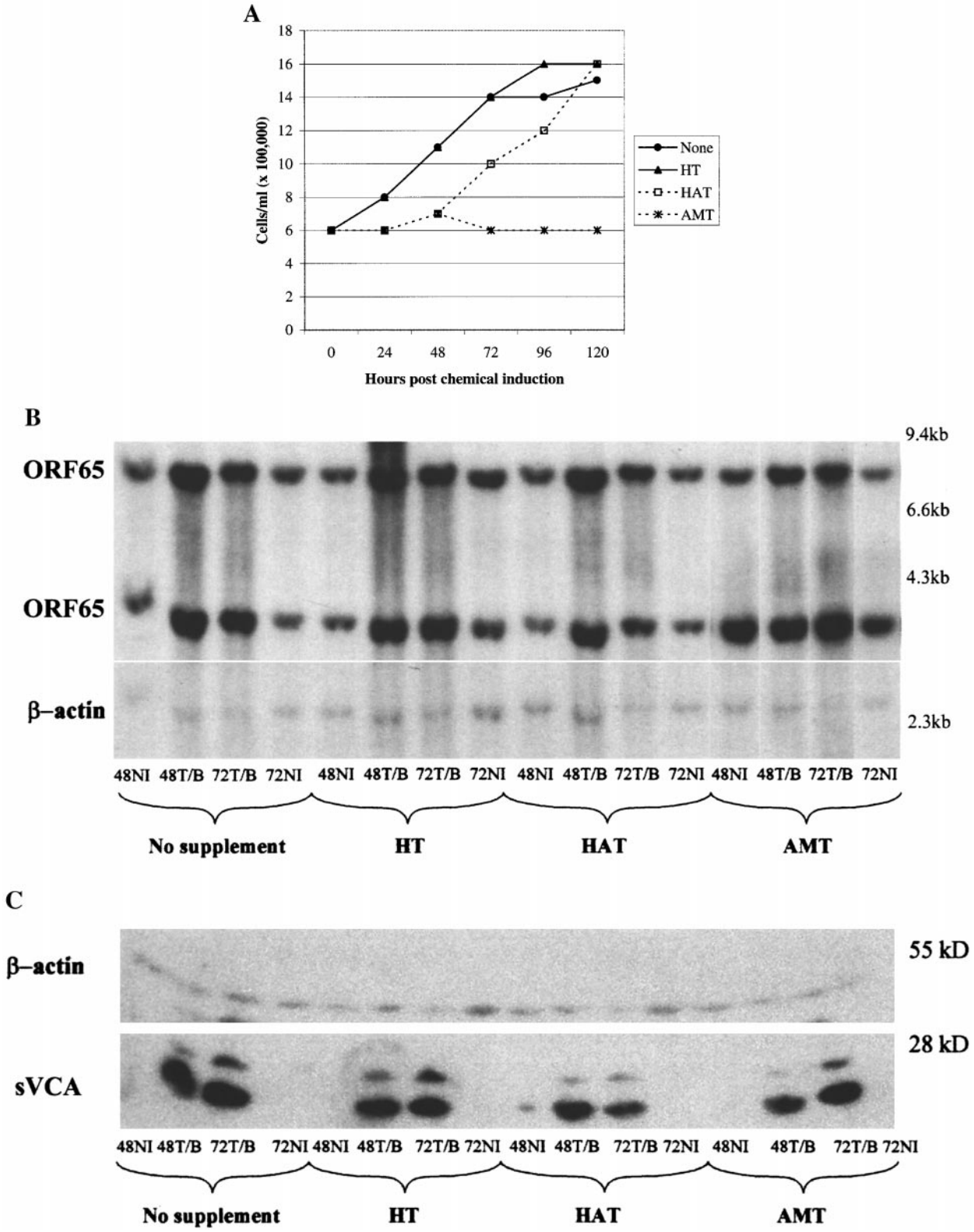

FIG. 8. Viral DNA content and expression of KSHV small capsid antigen (sVCA) in the presence of aminopterin (AMT). BC-3 cells were adapted to grow in HAT media (100 μM hypoxanthine [H], 16 μM thymidine [T], and 400 nM AMT). Adapted cells were washed extensively and subcultured. They were induced to enter the lytic cycle by treatment with TPA and butyrate in the presence of HT alone, HAT, AMT alone, or no supplement. Cell extracts were harvested at 48 and 72 h. (A) Growth curves for HAT-adapted BC-3 cells over a 120-h time course. (B) Total genomic DNA was extracted, digested with *Eco*RI, and separated on an agarose gel. The Southern blot was probed with a ^{32}P-labeled fragment of DNA containing ORF65 (*sVCA*) to measure viral DNA content. Loading was controlled for by probing the Southern blot with a ^{32}P-labeled human β-actin DNA fragment. (C) Cell extracts were separated by SDS–PAGE. The immunoblot was probed with rabbit antibody to sVCA, followed by ^{125}I-labeled protein A and mouse monoclonal antibody to human β-actin to control for loading. NI = noninduced. T/B = treated with TPA and butyrate. Some samples shown in (B) were rearranged to preserve continuity.

tent and in sVCA expression in the presence of AMT (Figs. 8B and 8C), these experiments indicated that concentrations of AMT that are known to inhibit both viral and cellular DHFR enzyme activity (Table 3), and were shown to inhibit proliferation of BC-3 cells (Fig. 8A), were unable to inhibit KSHV replication in cultured PEL cells. Thus we conclude that DHFR activity is not obligatory for viral replication *in vitro*.

DISCUSSION

In this report, we describe the properties of the DHFR enzyme encoded by KSHV, the most recently identified human herpesvirus. DHFR is known to be encoded by only two classes of DNA viruses, namely bacteriophage T4 and the rhadinovirus subgroup of the gammaherpesviruses, whose members include KSHV, *Herpesvirus saimiri*, and RRV (Searles *et al.,* 1999).

Based on nucleotide and amino acid homologies, the KSHV gene seems to have arisen from the primate cellular genome. However, the viral gene product has a 23-a.a. C-terminal appendage that so far does not appear to affect either enzymatic activity or nuclear localization. Unexpectedly, KSHV-DHFR as well as its human cellular counterpart were found to localize in the nucleus. Following chemical induction of the KSHV lytic cycle, viral enzyme was expressed to high levels, more than 10^5 copies per cell. However, based on studies with the folate inhibitor aminopterin, to which KSHV-DHFR is susceptible, the viral enzyme did not appear to be required for lytic viral DNA replication or viral late gene expression in cultured PEL cell lines.

Origin of the gammaherpesvirus DHFR gene

The nucleotide sequence of the KSHV-DHFR gene is 50% identical to that of the human DHFR gene; the HVS-DHFR gene is 76% identical to the human DHFR gene, and the RRV-DHFR gene is 63% identical to the human DHFR gene. The amino acid sequence of KSHV-DHFR is 50% identical, HVS-DHFR is 83% identical, and RRV-DHFR is 58% identical to human DHFR. These comparisons strongly suggest that the DHFRs of the three primate gammaherpesviruses and human DHFR share a common ancestor. It is likely that the viral genes arose from their cellular counterparts by reverse-transcription or recombination. However, the gene for KSHV-DHFR is located in the portion of the KSHV genome that also encodes cytokines and chemokines (Russo *et al.,* 1996), while the genes for HVS-DHFR (Albrecht *et al.,* 1992) and RRV-DHFR (Searles *et al.,* 1999) are located in a nonanalogous position of their respective genomes. This suggests that KSHV may have acquired its DHFR in an event that was distinct from that which occurred in HVS or RRV.

Moreover, KSHV-DHFR has a 23-a.a. C-terminal "tail" not found in HVS-, RRV-, or human DHFR. The amino acid sequence of the KSHV-DHFR 23-a.a. tail is not similar to any amino acid sequence in the protein data bases. However, there is a region downstream of the stop codon of the human DHFR gene which is 42% identical in nucleotide sequence to the region of KSHV-DNA encoding the 23-a.a. C-terminal tail of viral DHFR. This finding provides further support for the idea that KSHV-DHFR arose from cellular DHFR by retrotransposition and that this event was separate from the one(s) which led to the acquisition of DHFR by HVS and RRV.

DHFR localizes to the nucleus

We found, surprisingly, that epitope-tagged versions of both viral and cellular DHFR enzymes were located exclusively in the nucleus of transfected cells (Figs. 7B–7E). Nuclear localization of viral DHFR was independent of the epitope tag, the cell line, its state of confluence at the time of transfection, or the transfection method employed. These findings were unexpected because earlier immunofluorescence experiments made no suggestion that DHFR enzyme might be nuclear (Grill *et al.,* 1984). Early cell fractionation experiments showed that DHFR activity from rat liver was primarily located in the cytoplasm and mitochondria (Brown *et al.,* 1965). Studies using fluorescence-labeled MTX (F-MTX) showed that F-MTX, which was competent to bind DHFR, was exclusively in the cytoplasm (Kaufman *et al.,* 1978). Furthermore, other mammalian cellular enzymes such as TK and RR that are involved in nucleotide metabolism are located in the cytoplasm (Leeds *et al.,* 1985; Kucera and Paulus, 1986; Johansson *et al.,* 1997). However, although TS was once thought to be exclusively cytoplasmic (Kucera and Paulus, 1986), early and more recent evidence indicates that TS is also found in the nucleoplasm (Brown *et al.,* 1965; Samsonoff *et al.,* 1997).

The discovery of DHFR nuclear localization might lend some credence to the concept of a multienzyme "replitase" complex involved in dNTP synthesis (Prem veer Reddy and Pardee, 1980). This hypothetical complex comprised of DHFR, TK, TS, RR, nucleotide diphosphate kinase, and DNA polymerase is thought to form in the nucleus during S phase. It is postulated that the enzymes disassociate and leave the nuclear compartment after S phase (Prem veer Reddy and Pardee, 1980).

Our experiments, however, showed that human and viral DHFR were always present in the nucleus, even when the cells were confluent (i.e., in Go). Furthermore, similar experiments showed that KSHV-TS, -TK, and -RRα were exclusively in the cytoplasm. Moreover, in infected PEL cells viral DHFR was observed to be distributed diffusely through the nucleus and not in discrete granules, which might be expected to comprise a viral "replitase" complex or a compartmentalized site of viral DNA replication (de Bruyn Kops *et al.,* 1998).

Search for specific inhibitors of KSHV-DHFR

Cancer chemotherapy drugs such as aminopterin and methotrexate target cellular DHFR and, to a lesser extent, other folate-binding enzymes. The primary basis of this therapy is thought to be the high nucleotide requirement of rapidly dividing cancer cells. However, these drugs are not selective, for they also inhibit proliferation of rapidly dividing normal cells. The DHFR enzymes of some bacteria are also targets of antibiotics, such as trimethoprim. The basis of selective action of trimethoprim is that the inhibitor binds more tightly to the bacterial than to the mammalian DHFR enzyme. Therefore, we made preliminary efforts to identify a compound that would bind more avidly to KSHV-DHFR than to cellular DHFR (Table 3). KSHV-DHFR was less sensitive than rhDHFR to all of the five inhibitors studied. A possible reason for the high K_i of MTX for KSHV-DHFR is that in the viral enzyme, a methionine replaced phenylalanine at position 32. In human DHFR this amino acid plays a key role in binding MTX (Schweitzer *et al.,* 1989; Tsay *et al.,* 1990).

Although we have so far been unable to identify a compound that selectively inhibits viral DHFR, the use of a KSHV-DHFR inhibitor might find clinical applicability in combination with another class of antiviral drug. KSHV replication is sensitive to viral DNA polymerase inhibitors such as phosphoformate and chain terminators such as ganciclovir and penciclovir (Kedes and Ganem, 1997; Medveczky *et al.,* 1997). Therefore, these drugs might be used in combination with a DHFR inhibitor such as methotrexate in the treatment of Kaposi's sarcoma or other KSHV-associated diseases such as primary effusion lymphoma or multicentric Castleman's disease.

KSHV-DHFR is not essential for viral replication in cultured PEL cell lines

Our experiments with aminopterin showed that amounts of the inhibitor that block enzymatic activity *in vitro* and that inhibit cellular proliferation do not block KSHV lytic viral DNA replication or late gene expression (Fig. 8). Therefore, KSHV-DHFR is not necessary for viral replication in tissue culture. These results agree with experiments done in KSHV's counterpart HVS, in which HVS-DHFR was genetically eliminated without affecting HVS replication or immortalization (Desrosiers *et al.,* 1985, 1986).

What explains this result? The absence of BC-3 cellular proliferation in the presence of AMT excludes the possibility that HAT-adapted BC-3 cells have developed a cellular or viral DHFR enzyme that is resistant to the action of AMT. Since BC-3 cellular proliferation is inhibited when AMT is present and hypoxanthine and thymidine are absent, these nucleotide precursors may be limiting for cellular DNA replication but not for viral DNA replication. The nucleotide precursors for viral replication may come from intracellular pools, from the serum in the culture medium, or breakdown of cellular DNA during the lytic cycle of KSHV. Early experiments suggested that herpes simplex virus 2 derived some of its nucleotides from degradation of cellular DNA (Nutter *et al.,* 1985). However, host cell DNA is not incorporated into the genome of herpes simplex virus 1 (Prichard and Shipman, 1995).

Why does KSHV encode DHFR?

We have considered four possible answers to this question: (1) the viral DHFR enzyme is a structural component of the virion; (2) viral DHFR forms an essential component of a viral "replitase" complex consisting of virally encoded enzymes required for nucleotide metabolism; (3) the viral DHFR enzyme is required because the virus shuts off host cell macromolecular synthesis; and (4) a viral DHFR enzyme is needed because levels of the enzyme in the target cells susceptible to the virus *in vivo* are too low to meet the requirements of viral replication.

The first two hypotheses were provoked by studies of DHFR in bacteriophage T4. Early experiments suggested that the DHFR of T4 bacteriophage was incorporated into the basal tail plate of the phage (Wang and Mathews, 1989), but later immunoblot assays determined that this result was due to an artifact of the antibody preparation (Chen *et al.,* 1995). Using an immunoblot assay with antibodies to KSHV-DHFR, we were unable to detect any immunoreactive viral DHFR enzyme protein in virions produced from TPA-treated BC-3 cells (data not shown). T4-DHFR was shown to be a component of a multienzyme dNTP-synthesizing complex, as postulated by the replitase model (Wheeler *et al.,* 1996). However, our localization studies (Fig. 7) suggest that viral DHFR and TS are located in different subcellular compartments, a finding which is not consistent with the replitase model.

We have noted that KSHV lytic replication in BC-1 and HH-B2 PEL cells induced by sodium butyrate is accompanied by a dramatic decline in the abundance of cellular mRNAs (Miller *et al.,* 1997), including host-cell DHFR mRNA (Fig. 4C). This effect is selective; viral mRNAs, including that of KSHV-DHFR increase in abundance following butyrate treatment (Figs. 4A and 4B). While this effect could result from a viral mechanism that shuts off host-cell mRNA synthesis or degrades host-cell mRNAs, a more likely explanation is that the effect is directly caused by treatment with butyrate, independent of viral replication. A decrease in host-cell DHFR mRNA of similar magnitude was seen following butyrate treatment of B-cell lines lacking KSHV (not shown).

How might butyrate lead directly to inhibition of cellular DHFR mRNA expression? Expression of the hDHFR gene is regulated during the cell cycle. There is an increase in cellular DHFR transcription at the G_1/S-phase border (Slansky *et al.,* 1993). This increase is regulated by the transcription factor E2F. Histone deacetylase

(HDAC) inhibitors, such as butyrate and TSA, can activate the transcription of many genes, but they also arrest cells in the G_1 phase of the cell cycle (Gilbert and Weigle, 1993). When E2F is bound to retinoblastoma protein (Rb) during G_1, it acts as a repressor of cell-cycle genes such as DHFR (Hamel *et al.,* 1992; Weintraub *et al.,* 1995). In some cases, treatment with HDAC inhibitors such as butyrate and TSA does not relieve the E2F-Rb repression (Luo *et al.,* 1998). Thus, in KSHV-positive PEL cell lines, butyrate may exert opposing effects on the cellular and viral DHFR genes. The viral gene may be activated, perhaps as a result of hyperacetylation of the chromatinized viral genome, while the cellular gene may be repressed as the result of G_1 arrest and E2F repression caused by treatment with butyrate.

Cellular DHFR is a stable protein that persists after cellular DHFR mRNA is undetectable (Fig. 5) (Jackson and Huennekens, 1973; Domin *et al.,* 1982). This finding suggests that the level of cellular DHFR in cultured PEL cells is adequate to meet the dNTP requirements for KSHV DNA replication, even after the shut-off of host-cell mRNAs. However, a fuller exploration of this possibility should address the abundance of cellular versus viral DHFR enzyme in individual cells undergoing lytic viral replication. Since less than 25% of cultured PEL cells undergo lytic viral replication following application of a chemical-inducing stimulus, a decrease in the content of cellular DHFR in these cells might not be evident when viewed against the background of the cellular population as a whole.

In our view, the most likely explanation for the requirement for a virally encoded DHFR enzyme is that the virus may replicate in quiescent cells, such as lymphocytes or monocytes, that contain low levels of cellular DHFR and by inference low levels of FH_4 and dNTPs (Fig. 6). The level of cellular DHFR is at least 20-fold higher in cultured PEL cells than in primary PBMCs (Table 4). Therefore, cultured PEL cells are likely to contain pools of dNTPs that are considerably higher than those found in resting mononuclear cells. These pools may explain why inhibition of the cellular enzyme with AMT is not significantly detrimental to viral replication in PEL cells (Fig. 8). If KSHV were to use the cellular DHFR enzyme for its lytic replication in resting cells, it might be required to stimulate the cell into the late G_1 phase of the cell cycle when cellular DHFR is made (Slansky *et al.,* 1993). Thus far KSHV, unlike the related gammaherpesvirus EBV, which does not encode DHFR, has not been shown to be capable of activating infected lymphoid cells into DNA synthesis.

MATERIALS AND METHODS

Sequence analysis

All sequence comparison and analysis was done using BLASTP, BESTFIT, and GAP from the GCG software package.

Expression and purification of KSHV-DHFR

The KSHV-DHFR gene was cloned from genomic DNA from the BC-1 cell line using PCR. The 5′ primer used was 5′CTCGCGCCATGGATCCTACACTTTACTGTGTA3′. The 3′ primer used was 5′TGCCGGCTCTTCCGCACGAAGTCTCACTGAAGGGCG3′. The PCR product was ligated into the pCYB3 vector (New England Biolabs). This vector contains a 3′-autocleaving intein tag and a chitin-binding domain 3′ to the intein tag. The plasmid was transformed into BL21(DE3) *E. coli* (Novagen) for expression. The culture was induced overnight at room temperature in 0.4 mM IPTG. The bacteria were pelleted and resuspended in 30 mL buffer A (20 mM Tris–HCl, pH 8.0; 500 mM NaCl; 1 mM EDTA; and 0.1% Triton X-100) per liter of culture. The resuspended bacteria were lysed using a French press. The resulting lysate was centrifuged at 15,000*g* for 1 h. The supernatant (soluble fraction) was then applied to a 5-mL chitin bead column (New England Biolabs, Beverly, MA). The column was washed with 1 L of buffer A. After the wash, the column was flushed with buffer A containing 50 mM DTT and left overnight. Fractions were collected and protein concentration was determined by Coomassie protein assay (Pierce, Rockford, IL). DHFR activity assays were performed to determine the specific activity of the pure protein. To further purify the protein, the sample was first concentrated and then applied over a G-75 Sephadex gel filtration column connected to a Pharmacia LKB FPLC system. Peak fractions were collected and assayed for activity. The buffer used for FPLC was buffer A with 150 mM NaCl. The protein eluted at a retention volume of 30 mL, corresponding approximately to the molecular weight of 46 kDa, which implies that the enzyme is a dimer in solution. These fractions were examined electrophoretically on 12% polyacrylamide gels to determine purity (Fig. 2A). There were no detectable contaminants in the purest fractions.

The mutant that eliminated the C-terminal's 23 amino acids was cloned and purified as described earlier, except that the 3′ oligomer used for the PCR was 5′ACATCCGATGACGGTACCTTAAGTCACTTTTATGTA3′.

DHFR activity assay, determination of steady-state kinetics, and inhibition constant

A modified version of the standard DHFR activity assay was used to determine activity (Osborn and Huennekens, 1958). The components of the assay were as follows: 50 μM dihydrofolic acid (prepared by dithionite reduction of folic acid (Blakley, 1960)), 60 μM NADPH (Sigma, St. Louis, MO) and 12 μM β-mercaptoethanol in MATS buffer (25 mM 2-(*N*-morpholino)ethanesulfonate [MES], 25 mM sodium acetate, 50 mM Tris, and 100 mM NaCl). The decrease in absorbance at 340 nm was measured on a Beckman DU-70 spectrophotometer. Varying concentrations of protein (60 ng–20 μg) were used to

test for activity. FH_2 was added to initiate the reactions. Specific activities of the protein samples were determined by measuring the slope of the initial linear portion of the assay curve and then dividing by the extinction coefficient for the reaction and the concentration of the protein sample. The millimolar extinction coefficient at 340 nm used for the reaction $FH_2 + NADPH \rightarrow FH_4 + NADP^+$ was 12.8 ± 0.2 cm^{-1} (Appleman *et al.,* 1989). The K_m values of KSHV-DHFR, rhDHFR, and ntKSHV-DHFR for FH_2 were determined over a range of FH_2 concentrations that did not exhibit substrate inhibition (0.1–8 μM for the viral enzymes and 0.1–1 μM for rhDHFR) and 100 μM NADPH. K_m values for NADPH were determined by varying [NADPH] from 1 to 100 μM with a fixed concentration of FH_2 (8 μM for the viral enzymes, 1 μM for rhDHFR). Plots of velocity (V) vs. [substrate] were created and fit to a hyperbolic equation using Kaliedagraph software (Synergy Software), which generated the K_m and V_{max} values. Values of k_{cat} were determined from the equation $V_{max} = k_{cat}[E]_{tot}$.

MTX, TMP, AMT, and PYR were obtained from Sigma. PTX was generously donated by Dr. Debabrata Banerjee in the laboratory of Dr. Joseph R. Bertino (at Memorial Sloan-Kettering Cancer Center). For the K_i analyses, the concentration of each inhibitor was varied at fixed substrate concentrations. Plots of V versus [I] were then fit to the equation:

$$V = \frac{-A + \sqrt{A^2 + 4K_{i,app}[E]_{tot}}}{2[E]_{tot}} V_0$$

as described by Appleman *et al.* (1988a). $A = [I]_{tot} + K_{i,app} - [E]_{tot}$, $[I]_{tot}$ is the total concentration of inhibitor, $[E]_{tot}$ is the total concentration of enzyme (3–14 nM), $K_{i,app} = K_i(1 + [FH_2]/K_m)$, and V_0 is the reaction velocity without inhibitor. Fits were performed using the Kaliedagraph program.

Tissue culture

The BJAB and CI-16 cell lines were maintained in RPMI plus 8% FCS; the BC-1, BC-3, BCBL-1, and HH-B2 cell lines were maintained in RPMI plus 15% FCS; and 3 mM *n*-butyrate (butyrate) was used to induce all cell lines into lytic replication, except the BC-3 cell line (Miller *et al.,* 1997). For the BC-3 cell line, a combination of 3 mM butyrate and 20 ng/mL TPA was used to induce the lytic cycle.

Northern analysis

Whole-cell RNA extracts were prepared using the RNeasy kit from Qiagen (Chatsworth, CA). The hDHFR single-stranded oligonucleotide probe was complementary to an area of low nucleotide sequence identity in the 5′ region of the KSHV-DHFR gene. This oligonucleotide was 5′GTTCTGGGACACAGCGACGATGCAGTTTAGCGAA3′. The KSHV-DHFR oligonucleotide was complementary to the unique 3′ region of the KSHV-DHFR gene that encodes the unique 23-a.a. "tail" of the enzyme. This oligonucleotide was 5′CGAAGTCTCACTGAAGGGCGGGGTCGCGGGTCG3′. The RNA extracts were separated on a 1% agarose/10% formaldehyde gel. The RNA was transferred to a NYTRAN filter, which was probed with ^{32}P-radiolabeled DHFR olignucleotides. The amount of total cellular RNA contained in each sample was controlled for by probing the blots for RNaseP.

Western analysis

Rabbit anti-KSHV-DHFR antibodies were raised by injecting rabbits with viral DHFR that had been purified from *E. coli*. Rabbit anti-hDHFR antibodies were a generous contribution from Dr. Bruce Dolnick at SUNY Buffalo. Rabbit anti-hDHFR antibodies were also produced by injecting rabbits with rhDHFR expressed in *E. coli*. The rhDHFR expression vector was a generous contribution from Dr. Debabrata Banerjee in the laboratory of Dr. Joseph R. Bertino. The enzyme was purified as described previously using methorexate sepharose (Sigma) (Prendergast *et al.,* 1988).

Whole-cell extracts were sonicated, boiled, and loaded onto 12% polyacrylamide gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was then incubated with antibody. ^{125}I-labeled protein A was used to detect the primary antibody. Mouse monoclonal anti-β-actin antibody (Sigma) was used to control for loading.

Quantitation of KSHV-DHFR and hDHFR

Samples of purified KSHV-DHFR and rhDHFR were quantitated by hydrolysis and subsequent ion exchange chromatography at the protein analysis section of Yale's W. M. Keck Facility. These samples were used as standards for comparison with the amounts of DHFR present in cell extracts for quantitative, ^{125}I-protein A Western blotting. A phosphorimager was used to compare the amount of DHFR in the whole-cell samples to the amount of purified DHFR in the standards.

Isolation of CD3-depleted human PBMCs

Blood obtained by venipuncture was collected in a heparinized syringe. PBMCs were isolated over a Ficoll–Hypaque gradient (ICN) by spinning at 300*g* for 30 min. Cells were washed in PBS and resuspended at 2×10^7 cells/mL in PBS plus 2% fetal bovine serum. Antihuman CD3-conjugated magnetic beads (Dynal, Fort Lee, NJ) were added at manufacturer-recommended concentrations and the cell/bead suspension was incubated at 4°C on a nutator for 45 min. Beads were depleted three times using magnetic plates. Residual CD3-depleted cells were washed in RPMI before cell extracts were prepared for immunoblotting. The percentage of CD3-

positive cells was determined by cell-surface staining using anti-CD-3 FITC-conjugated antibodies (Sigma) and subsequent FACS analysis. After depletion, the percentage of CD3-positive cells was less than 3%.

Intracellular localization of rhDHFR, KSHV-DHFR, -TS, -TK, -RRα, -RRβ

rhDHFR, KSHV-DHFR, –TS, -TK, -RRα, -RRβ were cloned into the pCMV-tag2 vector (Stratagene, La Jolla, CA). The primers used to clone these genes from the BC-3 cell line DNA were as follows: rhDHFR 5′: 5′GAATTCGATATCCAAGCTTATGGTTGGTTCGCTA3′, rhDHFR 3′: 5′ACATCCGATGACCTCGAGTTAATCATTCTTCTC3′, KSHV-DHFR 5′: 5′TCCGGACTCAGATCTCGAGCTATGGATCCTACACTT3′, KSHV-DHFR 3′: 5′ACATCCGATGACGGTACCTTACGAAGTCTCACT3′, 24a.a. C-terminal KSHV-DHFR truncation mutant 3′: 5′ACATCCGATGACCTCGAGTTAAGTCACTTTTATGTA3′, KSHV-TS 5′: 5′GCATTATCGGATCCATGTTTCCGTTTGTACCT3′, KSHV-TS 3′: 5′GATATCCTCGAGCTATACTGCCATTTCCATACG3′, KSHV-TK 5′: 5′GATATCAAGCTTATGGCAGAAGGCGGTTTT3′, KSHV-TK 3′: GATATCCTCGAGCTAGACCCTGCATGTCTC3′, KSHV-RRα 5′: 5′GATATCGGATCCATGTCTGTCCGGACATTT3′, KSHV-RRα 3′: 5′GATATCCTCGAGCTACTGACAGACCAGGCACTC3′, KSHV-RRβ 5′: 5′GATATCGGATCCATGGATTCAGTTGATCGA3′, KSHV-RRβ 3′: 5′GATATCCTCGAGTCACAAATCGTCAGTCAC3′. These genes were cloned in-frame with an N-terminal flag tag. CHO cells were transfected with either vector alone or a flag-tagged gene using Lipofectamine (Gibco-BRL). Transfected cells were fixed on coverslips with 2% paraformaldehyde and incubated with mouse monoclonal antiflag M2 antibody (Stratagene). Cells were then incubated with goat anti-mouse IgG TRITC conjugated antibody (Sigma). Images were obtained using a Bio-Rad confocal microscope at magnification 630×.

Effects of AMT on KSHV replication in culture

To control for the toxic effects of AMT, BC-3 cells were slowly adapted to growth in HAT media (Gibco-BRL) consisting of 100 μM hypoxanthine (H), 16 μM thymidine (T), and 400 nM AMT. When the adapted cells were in logarithmic growth, they were washed three times in supplement-free media. Cells were then subcultured in media containing either no supplement, HT alone, HAT, or AMT (400 nM). Cells which were uninduced or treated with TPA and butyrate (as described earlier) were harvested after 48 and 72 h. For Southern analysis, the DNeasy kit (Qiagen) was used to extract genomic DNA. These samples were digested with *Eco*RI and separated on a 1% agarose gel. The samples were transferred to a nitrocellulose membrane and probed with a ^{32}P-labeled 735-bp *Bam*HI to *Xho*I fragment encompassing KSHV ORF65 from bp 112443 to 111931. Loading was controlled for by probing the blot with a ^{32}P-labeled 1.8-kb portion of β-actin cDNA. Whole-cell extracts prepared in SDS sample buffer were separated on 12% acrylamide gels. The resulting immunoblot was then probed with rabbit α-KSHV small capsid antigen (sVCA) antibodies (Lin *et al.,* 1997). Mouse monoclonal anti-β-actin antibodies were used to control for protein loading. ^{125}I-labeled protein A was used to detect the primary antibody. The resulting bands were quantified using a phosphorimager.

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